BIOLOGICAL CONTROL OF CROWN GALL DISEASE

CROSS-REFERENCE TO RELATED APPLICATIONS

Not applicable.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

[0001] This invention was made with United States Government support awarded by the United States Department of Agriculture under grant number USDA 00-CRHF-0-6055. The United States has certain rights in this invention.

BACKGROUND OF THE INVENTION

[0002] A genus of plant pathogenic bacteria is the genus *Agrobacteria. Agrobacterium* bacteria are naturally occurring plant genetic engineers. Members of the *Agrobacterium* genus natively have the ability to transfer a segment of DNA from a plasmid hosted by the bacterium into the genome of a cell of a living plant. The DNA transferred into the plant (the T-DNA) causes the plant cells to initiate two activities. One activity is to manufacture a class of chemicals, called opines, which can be metabolized by the bacteria as a food source. The other activity is to initiate the growth of a tumorous mass referred to as a crown gall.

[0003] *Agrobacterium* initiates crown gall growth in order to create a microenvironment, the crown gall, where the bacteria can thrive and multiply. Unfortunately, the crown gall itself saps metabolic energy from the plant that would otherwise be directed at producing vegetative growth or fruit, thus reducing the yield that would otherwise be produced by that plant. In some perennial plant species where the plant is long-lived, such as grapes, stonefruits and roses, the impact of crown gall disease can be significant. For example,

strains of the bacteria *Agrobacterium vitis,* which attacks vines, are the primary cause of grapevine crown gall, which is the most economically damaging bacterial disease of grape worldwide.

[0004] A number of different approaches exist for controlling plant diseases in commercial agriculture. One approach is based upon the application to plants or soil of chemical agents toxic or inimical to the disease-causing organism. A second approach is based on the development of plant varieties that are resistant to infection by the particular disease or strain of disease. One other approach is based on the use of a biological organism to control the disease-causing organism. This latter strategy is referred to as biocontrol.

[0005] Biocontrol agents control plant disease by secreting chemicals that act to inhibit or kill the disease-causing organism, or by simply occupying the ecological niche that would otherwise be available to the disease-causing organism. Some microorganisms are capable of inhibiting the growth of competing microbial strains through the use of toxins. If a bacterium can emit a broad-spectrum anti-bacterial toxin into its local environment, then that bacterium will have less competition in its ecological niche. As a result, many bacteria, and other microorganisms, have evolved genes for toxins. Often the plasmids that carry the genes encoding these toxins also carry genes conferring on its host immunity to that particular toxin. This is advantageous since obviously the toxin-secreting organism must have some mechanism to avoid the toxicity of its own toxin if the organism is to successfully populate the ecological niche it has cleared for itself.

[0006] Effective strategies to control crown gall by using biocontrol agents to control *Agrobacterium* growth are now known to exist. One problem associated with combating crown gall disease, however, is that different *Agrobacterium* species normally inhabit and attack plants in differing ecological micro-zones of the plant, as well as different plant species. For example, *Agrobacterium rhizogenes* normally lives in the root environment (the rhizosphere) of plants and attacks plant roots, while *Agrobacterium tumefaciens* normally attacks and infects plant stems or crowns. As a result, biocontrol strategies for crown gall disease must be focused on the micro-environment of the particular plant species and strains of *Agrobacterium* sought to be suppressed.

[0007] *Agrobacterium rhizogenes* strain K84, for example, is the most studied crown gall biological control strain and is commercially utilized for disease control on stone fruits worldwide. Strain K84 biological control is thought to be primarily due to the production

of two plasmid-encoded antibiotics, agrocins 84 and 434, encoded by genes on pAgK84 and pAgK434 respectively, each of which accounts for a portion of the observed disease control. Agrocin 84, an adenine analog, is effective against tumorigenic strains carrying nopaline/agrocinopine pTi plasmids, and requires the *accC* gene in the target strain for activity. Agrocin 434, a di-substituted cytidine analog, is effective against, and specific for, a broad range of *A. rhizogenes* strains. The commercial application of the K84 biological control system, however, is limited to stone fruits as pathogenic *Agrobacterium* strains of other crops are not inhibited by K84.

[0008] With respect to the *Agrobacterium vitis* strains, the causative agents of grapevine crown gall, no effective preventative control measures are commercially available. One prior attempt to use a biocontrol technique to combat crown gall in grape was based on the *Agrobacterium vitis* bacterial strain F2/5. Strain F2/5 is a non-tumorigenic *Agrobacterium* strain which may be applied to grapevines to occupy the ecological niches that might otherwise be occupied by tumorigenic strains. This strategy has met with some success, but the success is both grape-specific, i.e. does not work on other plant species, and is variable depending on the identity of the virulent *A. vitis* strain causing the disease. For example, F2/5 is ineffective on non-grapevine host plants, such as *Nicotiana glauca*, sunflower or tomato, and ineffective against various pathogenic *A. vitis* strains, such as CG78, as well as other *A. tumefaciens* biovars.

[0009] What is needed is a biocontrol agent with a broader target range to help control crown gall disease and, in particular, crown gall disease in grape plants.

BRIEF SUMMARY OF THE INVENTION

[0010] The present invention is summarized as a method for controlling crown gall disease in plants using an effective quantity of α-proteobacteria strain that produces trifolitoxin (TFX). The α-proteobacteria strain may be a biologically pure culture of an isolated TFX producing α-proteobacteria strain, as well as an α-proteobacteria strain genetically engineered to produce TFX. The present invention also includes the biocontrol agent of the above method, and a plant treated with the biological control agent.

[0011] The biocontrol agent is characterized as an α-proteobacteria strain genetically engineered to produce trifolitoxin (TFX). The α-proteobacteria strain employed may include any one of the many strains of *Agrobacterium*, including *Agrobacterium vitis* and,

in particular, *A. vitis* F2/5. The α-proteobacteria strain employed may be genetically engineered to produce TFX by introducing a genetic construct into the *Agrobacterium* so as to cause the *Agrobacterium* to carry and express the *tfx* operon from *Rhizobium*. The bacterium may also be genetically engineered to produce TFX by introducing a pT2TFXK plasmid into the *Agrobacterium*. The biocontrol agent may also be the strain *Agrobacterium vitis* F2/5 (pT2TFXK), ATCC Patent Deposit Designation PTA-2356.

[0012] It is an object of the present invention to create a biocontrol agent effective in inhibiting the occurrence of crown gall disease on plants and, in particular, in vine crop plant species.

[0013] It is a feature of the present invention that a novel and effective method to inhibit crown gall disease cause by many strains is made possible for the first time.

[0014] It is another feature of the present invention that a novel and effective method to inhibit crown gall disease above ground in vine crop plant species is now made possible.

[0015] Other object advantages and features of the present invention will become apparent from the following description and drawings.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

[0016] Fig. 1 is an illustration of the structure of the *tfx* operon.

[0017] Fig. 2 illustrates the putative chemical structure of the trifolitoxin peptide.

[0018] Fig. 3 graphically illustrates some of the results from the experiments described in the examples below.

DETAILED DESCRIPTION OF THE INVENTION

[0019] The present invention provides a method for controlling crown gall disease in plants using an effective quantity of an α-proteobacteria strain that produces trifolitoxin (TFX). The α-proteobacteria strain may be a biologically pure culture of an isolated TFX producing α-proteobacteria strain, as well as an α-proteobacteria strain genetically engineered to produce TFX. The present invention also includes the biocontrol agent of the above method, and a plant treated with the biological control agent.

[0020] Trifolitoxin is a peptide antibiotic natively produced by *Rhizobium leguminosarum* bv. *trifolii* T24, which natively inhabits the rhizosphere of bean plants. Antibiotic production and resistance functions are encoded by a seven-gene *tfx* operon and an unlinked

tfuA gene. Breil et al., "DNA sequence and mutational analysis of genes involved in the production and resistance of the antibiotic peptide trifolitoxin", *J. Bacteriol.* 175(12):3693-3702 (1993); and Breil et al., "A newly discovered gene, *tfu*A, involved in the production of the ribosomally synthesized peptide antibiotic trifolitoxin", *J. Bacteriol.* 178(14):4150-4156 (1996), incorporated herein by reference. TFX is derived from post-translational cleavage and modification of the *tfxA* gene product, and effectively inhibits growth of members of the α-proteobacteria, including strains of *Ochrobactrum, Rhodobacter, Rhodospeudomonas, Brucella,* and *Rhizobium.* TFX is highly specific for this group, as previously demonstrated by a lack of observable effect on non-Rhizobiaceae bacteria in the bean rhizosphere. Robleto et al., "Effects of bacterial antibiotic production on rhizosphere microbial communities from a culture-independent perspective", *Appl. Environ. Microbiol.* 74:5020-5022 (1998). A putative structure of active TFX is set forth in Figure 2.

[0021] We have discovered that it is possible to transfer a genetic construct encoding the production of TFX, and resistance to it, into an α-proteobacteria strain, such as *Agrobacterium*, and that such engineered bacteria can function as effective biocontrol agents for crown gall disease in plants. We have also discovered that TFX is inhibitory against a wide range of *Agrobacterium vitis* strains, including several strains that are poorly controlled by the strain, *A. vitis* F2/5. In addition, TFX production, expressed from the stable plasmid pT2TFXK, enhances the biological control activity of *A. vitis* F2/5 by making it both effective against strains that are not normally controlled by *A. vitis* F2/5, and by broadening its effective host plant range.

[0022] The biocontrol agent of the present invention is generally defined to include an α-proteobacteria strain that produces TFX. In one embodiment the biocontrol agent is an α-proteobacteria strain which has been genetically modified to produce TFX. Strains of α-proteobacteria are well known in the art. Suitable strains of α-proteobacteria are also described in Triplett et al., "Expression of *tfx* and Sensitivity to the Rhizobial Peptide Antibiotic Trifolitoxin in a Taxonomically Distinct Group of α-Proteobacteria Including the Animal Pathogen *Brucella abortus*", *App. Environ. Microbiol.*, 60(11):4163-4166 (1994), which is incorporated herein by reference. *Agrobacterium* is one selection of α-proteobacteria which have shown effectiveness in serving as biocontrol agents in accordance with the present invention. An example of one effective *Agrobacterium* species is *Agrobacterium vitis* and, in particular, the *A. vitis* strain F2/5.

[0023] The α-proteobacteria employed may be genetically engineered to produce TFX by introducing a nucleotide sequence into the bacteria that causes the bacteria to carry and express the *tfx* operon. The structure of the *tfx* operon is illustrated in Fig. 1. The operon includes seven genes designated *tfxA* through *tfxG*. The sequence for the *tfx* operon from *Rhizobium leguminosarum* bv. *Trifolii* is set forth in SEQ ID NO:1. The *tfxA* gene spans nucleotide bases 597 to 725, and its deduced amino acid sequence is set forth in SEQ ID NO:2. The *tfxB* gene spans nucleotide bases 794 to 1915, and its deduced amino acid sequence is set forth in SEQ ID NO:3. The *tfxC* gene spans nucleotide bases 1908 to 2978, and its deduced amino acid sequence is set forth in SEQ ID NO:8. The *tfxD* gene spans nucleotide bases 2982 to 4232, and its deduced amino acid sequence is set forth in SEQ ID NO:4. The *tfxE* gene spans nucleotide bases 4213 to 4971, and its deduced amino acid sequence is set forth in SEQ ID NO:9. The *tfxF* gene spans nucleotide bases 4968 to 5744, and its deduced amino acid sequence is set forth in SEQ ID NO:5. The *tfxG* gene spans nucleotide bases 5996 to 6781, and its deduced amino acid sequence is set forth in SEQ ID NO:6.

[0024] It is well known that copies of genes vary from strain to strain within a species. Such variations are referred to here as allelic variations. Accordingly, there are likely to be *tfx* operons in other bacterial species which may or may not have a sequence identical to SEQ ID NO:1 at each nucleotide. Such allelic variations to SEQ ID NO:1, as may exist, would not compromise the ability of the operon to effectively produce TFX in exactly the same manner as SEQ ID NO:1, and thus may be used in the practice of the present invention.

[0025] Many methods for introducing genetic constructs into bacteria so as to cause the bacteria to carry and express specific genes of interest are commonly known in the art, and may be employed in the present invention. For example, one may consider introducing a genetic construct containing the *tfx* operon into a bacterium so as to integrate at least one copy of the *tfx* operon into the bacterium's genome. Alternatively, one may consider introducing into a bacterium a plasmid that carries and expresses the *tfx* operon. In the latter case, the plasmid employed may include, with limitation, the pT2TFXK plasmid described in Triplett et al., "Expression of *tfx* and Sensitivity to the Rhizobial Peptide Antibiotic Trifolitoxin in a Taxonomically Distinct Group of α-Proteobacteria Including the Animal Pathogen *Brucella abortus*", *App. Environ. Microbiol.*, 60(11):4163-4166 (1994),

which is incorporated herein by reference. Although plasmid-borne traits are frequently unstable, pT2TFXK contains the RK2 plasmid-partitioning locus that confers a high degree of stability both *in vitro* and under field conditions. Stability of TFX expression would be beneficial when the biocontrol agent is inoculated onto the plant only at planting time, such as when roots are dipped in a bacterial suspension prior to planting.

[0026] In one embodiment, the biocontrol agent is the strain *Agrobacterium vitis* F2/5 (pT2TFXK) deposited with the American Type Culture Collection on August 8, 2000, and provided Patent Deposit Designation PTA-2356. Several features of F2/5(pT2TFXK) suggest that it is a safe agent for crown gall biological control. First, because of TFX's narrow range of toxicity, bacterial TFX production has little effect on non-target organisms. In addition, *A. vitis* itself is host-plant-specific, and has only been isolated from the grape rhizosphere, vineyard soils, and infested grapevines. Also, *A. vitis* survives poorly in bulk soil and non-grape rhizospheres, suggesting that F2/5(pT2TFXK) applied to grapevines would be unlikely to escape the vineyard or to affect the rhizospheres of other vineyard plants. This is in contrast to the commercially utilized *A. radiobacter* K84 strain, which has been shown to spread through fallow soil and to colonize the rhizosphere of many plant species. Further, pT2TFXK lacks *tra* genes and is therefore not self-mobilizable. A derivative of *A. radiobacter* K84, K1026, carries a Δ*tra* derivative of the self-transmissible pAgK84 is being used in Australia for control of crown gall.

[0027] The method of the present invention is generally defined to include the step of introducing onto a plant an amount of the TFX-producing α-proteobacteria strain which effectively inhibits or reduces crown gall growth as compared to a plant not treated with the TFX-producing α-proteobacteria. The TFX-producing α-proteobacteria strain may be an α-proteobacteria strain genetically engineered to produce TFX as described above, or a substantially pure culture of an α-proteobacteria strain that naturally produces TFX. A "substantially pure" culture shall be deemed to include a culture of TFX-producing α-proteobacteria containing no other bacterial species in quantities sufficient to interfere with the replication or TFX production of the culture, or sufficient to be detected by normal bacteriological techniques. Plants in which the present invention may be applied will generally include those plants susceptible to crown gall disease, such as grape plants, fruit trees, and rose plants.

[0028] It is broadly intended within the scope of the present invention that the biocontrol agent will be applied to the plant, or inoculated into the soil with the plant or plant seeds so that a culture of the biocontrol agent will develop near or within the susceptible tissue of the plant as it grows. To facilitate this, it is preferred that the biocontrol agent, preferably diluted with a suitable extender or carrier, either be applied to the seeds or plants prior to planting or introduced into the furrows when the seeds or plants are planted. Alternatively, the biocontrol agent may be prepared with or without a carrier and sold as a separate inoculant to be applied directly to the plant or inserted directly into the furrows into which the seed or plant is planted. Such processes are generally well known in the art.

[0029] One advantageous technique may include applying the biocontrol agent to the plant or seeds through the use of a suitable coating mechanism or binder prior to the seeds or plants being sold into commerce for planting. The process of coating seeds and plants is generally well known to those skilled in the art. For example, the biocontrol agent may be mixed with a porous, chemically inert granular carrier as described by U.S. Patent No. 4,875,921, which is incorporated herein by reference.

[0030] Whether or not the biocontrol agent is coated directly on the seed or plant, the biocontrol agent is preferably diluted with a suitable carrier or extender so as to make the culture easier to handle and to provide a sufficient quantity of material so as allow easy human handling. For example, a peat based carrier may be used as described by Bosworth et al, "Alfalfa yield response to inoculation with recombinant strains of *Rhizobium meliloti* carrying an extra copy of *dct* and/or modified *nif*A expression," Appl. Environ. Microbiol., 60:3815-3832 (1994), incorporated herein by reference. In addition, it has been discovered that perlite, vermiculite and charcoal materials are suitable carrier substances. It is believed that many other non-toxic and biologically inert substances of dried or granular nature are also capable of serving as carriers for the biocontrol agent.

[0031] The density of inoculation of the biocontrol agent onto the plant or plant seed, or into the furrows, should be sufficient to populate the region of the seed or plant, or the sub-soil region adjacent to the roots of the seed or plant, with viable bacterial growth. An effective amount of biocontrol agent should be used. An effective amount is that amount sufficient to establish sufficient bacterial growth so that infection by crown gall inducing bacteria is inhibited or diminished as compared to infection by crown gall inducing bacteria in the absence of the biocontrol agent.

[0032] The project, results of which are described below, began as an effort to develop a strain of bacteria that could be used as a biocontrol agent for controlling crown gall disease in vine crops, such as grape. The thought was to introduce into an *Agrobacterium* species the ability to produce the TFX antibiotic so as to control tumorigenic *Agrobacterium* species that might otherwise prey upon susceptible plants. It was unknown at the initiation of this effort whether or not the TFX toxin would be effective within crown-gall susceptible plant tissue, as well as above ground environments exposed to sunlight, air, and other destabilizing agents. The toxin is normally found only in the rhizosphere of growing plants, which had been the only previous environment in which the TFX toxin had been demonstrated to be effective. We discovered that production of the TFX toxin can be successfully engineered into *Agrobacterium* species for use as a biocontrol agent to control the spread of tumorigenic *Agrobacterium* species within crown-gall susceptible tissue.

[0033] It was uncertain as to whether the species of *Agrobacterium* responsible for crown gall disease were susceptible to the TFX toxin. As described below, tests conducted to determine whether or not the *Agrobacterium vitis* strains were susceptible to TFX demonstrated that there was a level of susceptibility, but it was lower than might be expected upon previous TFX sensitivity measurements with other *Agrobacterium* species. In tests with *Rhizobium leguminosarum* T24 colonies only relatively small zones of inhibition were observed around the *Rhizobium* producing TFX. One *Agrobacterium* strain, biocontrol strain F2/5, was found to be TFX-resistant. Nevertheless, it was found that when the plasmid encoding the production of TFX was introduced into the *A. vitis* strain F2/5, an effective crown-gall biocontrol agent was created which was effective against most tumorigenic strains of *A. vitis* when co-inoculated with the tumorigenic strain. The biocontrol agent was found to be effective when the ratio of the biocontrol agent to the tumorigenic strains was at a ratio of 1 to 1 or higher.

[0034] Prior to this work it was also not clear whether TFX production would be effective at inhibiting galling by tumorigenic *Agrobacterium in planta* because TFX is rapidly degraded *in situ*. Previous studies have observed the degradation of antimicrobial peptides such as cecropin B and attacin E in plant apoplastic fluids. This was likely due to apoplastic proteinases. As a result, expression of antimicrobial peptides in plants had mixed results for enhancing disease resistance. For example, cecropin expression in transgenic tobacco did not confer resistance to *P. syringae* pv *tabaci*, likely due to low

apoplastic peptide concentrations due to proteolysis. Jones and Kerr, "*Agrobacterium radiobacter* strain K1026, a genetically engineered derivative of strain K84 for biological control of crown gall", *Plant Disease* 73:15-18 (1989); Mills et al., "Evidence for the breakdown of cecropin B by proteinases in the intercellular fluid of peach leaves", *Plant Sci.* 104:17-22 (1999).

[0035] It was also believed that TFX production would be difficult to engineer in plants due to the complex mechanism by which active TFX is derived from *tfxA*. We discovered that *Agrobacterium rhizogenes* is capable of producing TFX by addition of the pT2TFXK plasmid containing the *tfx* operon but not *tfuA*. This discovery suggested that TFX production by crown gall biological control strains of *Agrobacterium*, such as *A. rhizogenes* K84 and *A. vitis* F2/5, may be enhanced by TFX production, and provide excellent delivery vehicles for TFX to the infection site.

[0036] There are additional benefits obtained by the production of the TFX toxin in the *A. vitis* strain F2/5. For example, biocontrol is extended to other non-grape vine hosts of *Agrobacterium,* such as *Nicotinia glauca*. The enhancement of the biocontrol F2/5 strain by virtue of the ability to produce TFX extends the ability of the F2/5 strain to control *A. vitis* strains on grapevine that it could not otherwise control without the ability to produce TFX. This result demonstrates that TFX production would enhance crown gall biocontrol for all other biocontrol strains, particularly of *Agrobacterium* strains, which can be used on other host plants. The ability to produce TFX in *Agrobacterium* offers the ability to confer biocontrol upon a strain as long as the producing strain is present in excess of the tumorigenic strain. High ratios of biocontrol to pathogen strain can easily be achieved in field situations by dipping the roots of planting stock in suspension of the biocontrol strain or by direct application of the bacterial suspensions of the biocontrol strain to the planting beds or to the plants themselves.

[0037] Thus, the ability to enhance the biocontrol status of *Agrobacterium* strains is not limited to particular exemplary strains of *Agrobacterium* described above and in the examples below. The results demonstrate that the TFX phenomenon of pathogen inhibition can be achieved in environments other than the rhizosphere and that the strategy works well in above ground environments. It thus becomes possible to transfer this toxin producing activity to any *Agrobacterium* strain sought to be used as a biocontrol agent. The plasmids

described below are suitable and appropriate for introducing such activity into other crown gall forming bacterial strains, including other *Agrobacterium* strains.

[0038] While the examples set forth below are executed in *Agrobacterium vitis*, the same technique is anticipated to work in other crown gall forming bacterial strains, such as other strains of *Agrobacterium*. The examples below are intended to only be illustrative of the aspects of the present invention, and neither serve to limit or diminish the scope of the present invention.

EXAMPLES

Bacterial and plant growth conditions, strain construction

[0039] Bacterial strains investigated are listed in Table 1 below. The *A. vitis* strains (without pT2TFXK or pT2TX3K) were obtained from Dr. T.J. Burr, Cornell University. Bacteria were grown on BSM agar medium at 27°C. *A. vitis* F2/5(pT2TFXK) and F2/5(pT2TX3K) were constructed by triparental mating using standard procedures. Transconjugants were selected on BSM medium amended with 50ppm kanamycin. Trimethoprim (10ppm) was added to counterselect the *E. coli* donor and helper strains. Strains containing the plasmids pT2TFXK and pT2TX3K were grown for routine propagation on BSM amended with 50 ppm kanamycin. Prior to use in making inoculum suspensions for biological control assays these strains were grown overnight on BSM agar without kanamycin. The plasmids pT2TFXK and pT2TX3K both contain the full operon encoding the TFX peptide toxin, including genes *tfxA* through *tfxG*.

[0040] Plants (*Nicotiana glauca*) were grown in the greenhouse with supplemental illumination and fertilized as needed with a nutrient solution called CNS containing 2 mM $CaCl_2.2H_2O$, 0.5 mM $MgSO_4.7H_2O$, 2 mM KCl, 0.4 mM KH_2PO_4, 2.5 mM NH_4NO_3, 0.065 mM $FeSO_4.7H_20$, 2.3 μM H_3BO_3, 0.9 μM $MnSO_4.H20$, 0.6 μM $ZnSO_4.7H_2O$, 0.1 μM $NaMoO_4.2H_2O$, 0.11 μM $NiCl_2.6H_20$, 0.01 μM $CoCl_2.6H_2O$, 0.15 μM $CuSO_4.5H_2O$.

Table 1. Bacterial Strains

Strain	Characteristics
Rhizobium	
T24	
T24 Tn5::*tfxB*	
CE3 (pT2TFXK)	
CE3 (pT2TX3K)	
Agrobacterium vitis	
F2/5	
F2/5 (pT2TFXK)	
F2/5 (pT2TX3K)	Plasmid contains *tfxA* deletion, non-Tfx
CG561	Non-tumorigenic, non-biocontrol on grapevine
CG561 (pT2TFXK)	Contains Tfx-encoding plasmid, produces TFX
CG561 (pT2TX3K)	Plasmid contains *tfxA* deletion, non-Tfx producing strain
CG49	Tumorigenic, nopaline-type pTi, controlled by F2/5 on grape
CG78	Tumorigenic, vitopine-type pT1, not controlled by F2/5 coinoculation
K306	Tumorigenic, octopine-type pT1, controlled by F2/5 on grape
CG106	Tumorigenic
CG113	Tumorigenic
CG435	Tumorigenic

***In vitro* antibiosis assay**

[0041] *Agrobacterium vitis* strains (Table 1) were tested for sensitivity to trifolitoxin (TFX). Ten µl of the trifolitoxin-producing strain *Rhizobium leguminosarum* T24, or its Tfx-derivative, were spotted directly from frozen stocks onto BSM agar plates (Difco). The bacteria were allowed to grow for two to three days at 27°C to allow TFX to accumulate in the medium. These plates were then sprayed with a light mist of either the highly-TFX sensitive *Rhizobium* 128Cl (positive control) or an *Agrobacterium vitis* test strain using a Preval spray gun (Precision Valve Company part #267).

[0042] Test-strain spray suspensions were made by suspending loops full of bacteria into 15 ml sterile distilled water until the suspension was barely visibly turbid. Sprayed plates were incubated for 2-4 days at 27°C. When growth of the test strain was apparent on the TFX non-producing strain plates, the plates were scored for zones of no growth around the TFX producing strain. Lack of a zone around the TFX non-producing strains indicated that zone formation was not due to factors other than TFX production. The same procedure was

used with *Rhizobium* CE3 (pT2TFXK) and (PT2TX3K) as the TFX-producing and non-producing strains, respectively.

[0043] As expected based on previous results, the tested *Agrobacterium vitis* strains were sensitive to TFX-producing strains. However, the level of susceptibility was lower than predicted based upon previous TFX sensitivity measurements with CG-48 and CG-74. No zones of *A. vitis* growth inhibition were observed around *R. leguminosarum* T24 colonies, and only relatively small zones were observed around *R. etli* CE3(pT2TFXK), which produces more TFX than T24. Furthermore, one *A. vitis* strain, F2/5, was TFX-resistant.

[0044] Because *A. vitis* F2/5 produces an agrocin to which most of the tumorigenic strains are sensitive, the effect of TFX on *A. vitis* was assessed against TFX-producing and non-producing *Rhizobium* strains. None of the tested strains were sensitive to *R. leguminosarum* T24, which produces relatively low amounts of TFX. All of the *A. vitis* strains except for F2/5 were sensitive to *R. etli* CE3(pT2TFXK) as evidenced by zones of growth inhibition around the CE3(pT2TFXK) colonies. *A. vitis* growth was not inhibited by a non-TFX metabolite or nutrient competition by CE3(pT2TFXK) as evidenced by the lack of a zone around the near-isogenic *tfxA* mutant CE3(pT2TX3K) colony.

Evidence for TFX production by *A. vitis* strain F2/5(pT2TFXK).

[0045] *A. vitis* strain F2/5 (pT2TFXK) was tested to determine if the strain was producing TFX. The assay was performed as described above with F2/5 (pT2TFXK) cultured in a single colony in the center of the plate. One day after growth at 28°C, the plates were sprayed with a dilute suspension of ANU794(pT2TX3K) or ANU794.

[0046] It was observed that strain F2/5(pT2TFXK) inhibited TFX-sensitive *R. leguminosarum* bv. trifolii ANU794 but had no effect on ANU794 following addition of the TFX resistance genes provided by pT2TX3K. Plasmids pT2TFXK and pT2TX3K confer resistance to TFX, tetracycline, and kanamycin with pT2TFXK also providing TFX production to a host strain. However, since strains F2/5 and F2/5(pT2TX3K) produced no zones of inhibition against ANU794 or ANU794(pT2TX3K), F2/5(pT2TFXK) is producing TFX.

***In planta* biological control of crown gall by TFX-producing strains**

[0047] *Agrobacterium vitis* strains were suspended in sterile distilled water prior to the determination of colony forming units (CFU) per ml. These suspensions were adjusted to OD_{650} 0.10 (approximately 10^8 CFU/ml) using a Shimadzu UV-160 spectrophotometer and sterile distilled water, and stored until inoculation at 4°C. Actual inoculum viability and cell density were measured by dilution plating on BSM agar medium on the day that plants were inoculated.

[0048] Prior to inoculation, tumorigenic strains were diluted 10-fold with sterile distilled water to approximately 10^7 CFU/ml. Biological control stocks were left undiluted, or diluted 10-fold (for CG49 and CG435 experiments) or 100-fold (for CG78 experiments). Thus, the CFU ratios were approximately 10:1, and 1:1 or 1:10 avirulent:tumorigenic strain. Immediately prior to plant inoculation tumorigenic strains were mixed 1:1 (vol:vol) with the appropriate biological control test strain. Positive controls were diluted 1:1 (vol:vol) with water. Thus, all plant inocula contained approximately 5 x 10^6 CFU/ml of the tumorigenic strains.

[0049] Plants (*Nicotiana glauca*) were inoculated by wounding the stem with a dissecting needle. Three or four inoculations were made per inoculum mixture on each of two plants. Thus, each of the two experiments included 6 to 8 repetitions per treatment. A 5 µl drop of bacterial suspension was placed on the wound and allowed to air dry. Inoculation sites were wrapped loosely with Parafilm (American National Can) for 1 week post-inoculation. Gall diameter perpendicular to the stem was measured 4 to 7 weeks post-inoculation using a caliper, and all measurements were included for statistical analysis. Results were analyzed using ANOVA at the $\alpha = 0.05$ level of significance.

[0050] As expected, F2/5 did not inhibit galling by tumorigenic *A. vitis* strains on *N. glauca*. A 10:1 ratio of *A. vitis* F2/5(pT2TFXK):pathogen caused a significant reduction in mean gall size relative to the TFX non-producing controls on *N. glauca* stems for all three tested tumorigenic strains. (Fig. 3.) High concentrations of F2/5(pT2TFXK) also reduced gall incidence for CG435 and CG78, but not for CG49. (Table 2.) A 1:1 ratio of F2/5(pT2TFXK):CG435 also resulted in a significant reduction in gall size and in gall incidence compared to controls. A 1:1 ratio of F2/5(pT2TFXK):CG49 or F2/5(pT2TFXK):CG78 did not affect either incidence of galls or reduce gall size. Similarly, an excess (a 1:10 ratio) of any of the virulent strains to F2/5(pT2TFXK) resulted

in a high incidence of disease and large gall size.

Table 2. Effect of (pT2TFXK) on Gall Incidence

	Tumorigenic Strain[a]		
Biocontrol Strain	CG49	CG435	CG78
F2/5	6/6	6/6	6/6
F2/5 (pT2TFXK)	6/6	0/6	1/8

a. Inoculations performed at 10:1 ratio of biocontrol:pathogenic strain. Presence of galls was scored visually by comparison to an uninoculated negative control one month post-inoculation.

[0051] Two principle benefits of TFX production by F2/5 were demonstrated. Biological control was extended to the non-grapevine host *N. glauca*, and biological control was extended to a strain that F2/5 failed to control (CG78) on grapevine. These effects are due to TFX production as demonstrated by the lack of efficacy of F2/5 against CG49, CG435, and CG78, and by the lack of efficacy of the near-isogenic TFX-non-producing F2/5(pT2TX3K) against CG78. TFX also provided biological control when the TFX-producing strain was present in excess of the tumorigenic strain. Thus, F2/5(pT2TFXK) effectively inhibited galling by all three tested tumorigenic strains when co-inoculated in approximately 10-fold excess.

[0052] At 1:1 or 1:10 inoculum ratios of F2/5(pT2TFXK):pathogen biological control was reduced or lost. This could be overcome by using high ratios of biological control:pathogen in the field, which should be easily achieved by dipping the roots of planting stock in suspensions of the biological control strain, or by directly applying the bacterial suspension to the planting bed.

[0053] TFX was also discovered to be inhibitory towards all tested species of *Agrobacterium*. These results suggested that TFX production would enhance crown gall biological control for other biological control strains, such as *A. rhizogenes* K84, and on other host plants, especially where a mixed inoculum of different tumorigenic *Agrobacterium* species occurs.

[0054] The effect of TFX production on gall size and the effective inoculum ratios are also illustrated graphically in Fig. 3. Gall diameter in millimeters perpendicular to the stem was measured one month post-inoculation. Wound sites were inoculated with 5 µl of mixed bacterial suspensions. Each inoculum mixture was inoculated into three wound sites on each of two plants, for a total of six inoculations per treatment.